Estradiol increases IP₃ by a nongenomic mechanism in the smooth muscle cells from the rat oviduct

Patricia Reuquén¹,², María L Oróstica¹,², Israel Rojas¹,², Patricia Díaz¹,², Alexis Parada-Bustamante³ and Pedro A Orihuela¹,²

¹Laboratorio de Inmunología de la Reproducción, Facultad de Química y Biología, Universidad de Santiago de Chile, ²Centro para el Desarrollo en Nanociencia y Nanotecnología-CEDENNA and ³Instituto de Investigaciones Materno-Infantil, Universidad de Chile, Alameda 3363, Casilla 40, Correo 33 Santiago, Chile

Correspondence should be addressed to P A Orihuela; Email: pedro.orihuela@usach.cl

Abstract

Estradiol (E₂) accelerates egg transport by a nongenomic action, requiring activation of estrogen receptor (ER) and successive cAMP and IP₃ production in the rat oviduct. Furthermore, E₂ increases IP₃ production in primary cultures of oviductal smooth muscle cells. As smooth muscle cells are the mechanical effectors for the accelerated oocyte transport induced by E₂ in the oviduct, herein we determined the mechanism by which E₂ increases IP₃ in these cells. Inhibition of protein synthesis by Actinomycin D did not affect the E₂-induced IP₃ increase, although this was blocked by the ER antagonist ICI182780 and the inhibitor of phospholipase C (PLC) ET-18-OCH₃. Immunoelectron microscopy for ESR1 or ESR2 showed that these receptors were associated with the plasma membrane, indicating compatible localization with E₂ nongenomic actions in the smooth muscle cells. Furthermore, ESR1 but not ESR2 agonist mimicked the effect of E₂ on the IP₃ level. Finally, E₂ stimulated the activity of a protein associated with the contractile tone, calcium/calmodulin-dependent protein kinase II (CaMKII), in the smooth muscle cells. We conclude that E₂ increases IP₃ by a nongenomic action operated by ESR1 and that involves the activation of PLC in the smooth muscle cells of the rat oviduct. This E₂ effect is associated with CaMKII activation in the smooth muscle cells, suggesting that IP₃ and CaMKII are involved in the contractile activity necessary to accelerate oviductal egg transport.


Introduction

The canonical pathway by which estradiol (E₂) affects its target cells comprises binding to estrogen receptors (ER) and modification of gene expression and protein synthesis (Nilsson et al. 2001). However, some E₂ effects cannot be blocked by inhibitors of transcription or translation, or are too rapid to be due to changes in gene expression. These features do not appear compatible with the classical genomic actions and are termed nongenomic (Lössel et al. 2003, Lössel & Wheling 2003). E₂ nongenomic actions often involve the activation of G protein-α inhibitory (Gαi), stimulation of intracellular signal transduction pathways, including the generation of second messengers such as cAMP and IP₃, and activation of protein kinase A (PKA) or phospholipase C (PLC) in the E₂-target cells (Nadal et al. 2001, Wyckoff et al. 2001, Accocia et al. 2005, Hill et al. 2010).

In the rat, a single injection of E₂ on day 1 of the cycle or pregnancy shortens oviductal transport of eggs from the normal 72–96 h to <24 h (Ortiz et al. 1979). We have previously demonstrated that RNA and protein synthesis inhibitors did not block E₂-induced oviductal egg transport acceleration in unmated rats indicating that E₂ accelerates oviductal egg transport by a nongenomic mechanism (Orihuela et al. 2001). This E₂ nongenomic pathway involves a previous conversion of E₂ to methoxyestradiols through the activation of catechol-O-methyltransferase (COMT) (Parada-Bustamante et al. 2007, 2010), ER and adenylyl cyclase (AC) (Orihuela et al. 2003), and sequential production of cAMP and IP₃ (Orihuela et al. 2003, 2006, 2013).

The rat oviduct is mainly composed of an intrinsic layer of smooth muscle fibre, and an innermost highly folded mucosa formed by epithelial and stromal cells, the endosalpinx (reviewed in Croxatto (2002)). Transport of oocytes along the oviduct depends on the interaction between the secretory activity of the epithelial cells and the contractile activity of the smooth muscle cells (Moore & Croxatto 1988a,b, Ríos et al. 2007). The regulation of muscular motility is influenced by E₂ and requires activity of adrenergic nerves (Helm et al. 1982), nitric oxide (Perez Martinez et al. 2000), endothelin (Parada-Bustamante et al. 2012), oxytocin (Jankovic et al. 2001) and prostaglandins (Wijayagunawardane et al. 2003). These factors activate intracellular
signalling mainly associated with Ca\textsuperscript{2+}, cAMP or IP\textsubscript{3} (Jankovic et al. 2001, Barrera et al. 2004, Mohan et al. 2012). In this context, we have recently shown that, in the epithelial cells of the rat oviduct, E\textsubscript{2} increased cAMP production between 3 and 6 h, although IP\textsubscript{3} levels were not affected. Moreover, E\textsubscript{2} increased cAMP in the oviductal epithelial cells by a nongenomic mechanism that requires coupling between ESR1 and G\alpha\textsubscript{i} and stimulation of AC (Oróstica et al. 2014). Previous research has also shown that E\textsubscript{2} increased IP\textsubscript{3} levels in primary cultures of smooth muscle cells from the rat oviduct (Oróstica et al. 2014).

As smooth muscle cells are the mechanical effectors for the accelerated oocyte transport induced by E\textsubscript{2} in the oviduct (Croxatto 2002), this work determined the mechanism by which E\textsubscript{2} increases IP\textsubscript{3} in primary cultures of rat oviductal smooth muscle cells. Thus, we examined the effect of E\textsubscript{2} on the IP\textsubscript{3} levels in the smooth muscle cells under conditions in which protein synthesis, ER, G\alpha\textsubscript{i} or PLC activity were blocked by selective inhibitors. The subcellular localization of ESR1 and ESR2 as well as the effect of selective agonists for ESR1 or ESR2 on the IP\textsubscript{3} level was evaluated in the smooth muscle cells. Furthermore, expression of G\alpha\textsubscript{i} in the oviductal smooth muscle cells was also determined. Finally, the effect of E\textsubscript{2} on the activity of the enzyme associated to muscle contraction calcium–calmodulin protein kinase II (CaMKII) was determined in the primary cultures of smooth muscle cells.

Materials and methods

Animals

Locally bred Sprague-Dawley rats weighing 200–260 g were used. Animals were kept under controlled temperature (21–24 °C), and lights were on from 0700 to 2100 h. Water and pelleted rat chows were supplied ad libitum. Female mature rats were used in the estrous stage. The phases of the estrous cycle were determined by daily vaginal smears (Turner 1961) and all females were used after showing 2 consecutive 4-day cycles. The Ethical Committees of the Universidad de Santiago de Chile and the National Fund of Science (CONICYT-FONDECYT 1110662) approved the protocols for the care and manipulation of the animals.

Culture of primary smooth muscle cells from rat oviducts

For each replicate, 12 oviducts from six rats were excised and placed in pre-warmed Hanks’s solution (Sigma Chemical) at pH 7.4. The whole oviduct was cut into small (4–8 mm\textsuperscript{2}) pieces in Hank’s solution and then the smooth muscle cells were mechanically removed from the rest of the tissue and treated with Collagenase, Type I (Invitrogen) for 1 h to further disaggregation of the cells. The cell suspension was centrifuged at 1200 g during 5 min, washed, and seeded into six-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) in DMEM/High Modified medium with 4.0 mM l-glutamine and 4.5 g/l glucose free of Phenol Red (Cat. No. SH30284.02, HyClone, Thermo Scientific, Waltham, MA, USA) supplemented with 10% (v/v) foetal bovine serum (Cat. No. SH30396.03, HyClone), 1 mM sodium pyruvate and 100 U/ml penicillin and 100μg/ml streptomycin. Smooth muscle cells were incubated at 37 °C in an atmosphere of 5% (v/v) CO\textsubscript{2} for at least 7 days to reach 75–80% confluence and their purity verified by immunofluorescence staining for cytokeratin (marker of epithelium cells), vimentin (marker of fibroblasts) or α-actin (marker of smooth muscle cells) antibodies.

Extraction and measurement of IP\textsubscript{3}

Primary smooth muscle cell cultures were sonicated in 100 μl of ice-cold 1 M trichloroacetic acid (TCA) and an aliquot was taken to measure protein concentration by the Bradford assay using BSA dissolved in 1 M TCA as standard (Bio-Rad). The remaining homogenate was then centrifuged for 10 min at 10000 g at 4 °C. The pellet was discarded and the supernatant was incubated for 15 min at room temperature. TCA was removed from the supernatant with 0.5 ml of a solution 1,1,2-Trichloro-trifluoroethane (TCTFE, Sigma)-Trioctylamine (Sigma), 3:1 (v/v). Levels of IP\textsubscript{3} were determined using IP\textsubscript{3} [\textsuperscript{3}H] radioreceptor assay Kit, Cat. No NEK064 (NEN Life Science Products, Boston, MA, USA). This kit is based on competition between-naradiaoactive IP\textsubscript{3} and a fixed quantity of [\textsuperscript{3}H]-IP\textsubscript{3}, for a limited number of calf cerebellum IP\textsubscript{3} receptor binding sites. This allows the construction of a standard curve and the measurement of IP\textsubscript{3} levels in unknown samples.

Western blot

Polyclonal antibodies that recognize the phosphorylated state of CaMKII on Thr286 (anti-phospho-CaMKII, Cell Signaling Technology, Beverly, MA, USA) or total CaMKII (anti-CaMKII,
Abcam, Cambridge, UK) were used to assess activation of CaMKII. Smooth muscle cells were processed by duplicate to determine the activity of the CaMKII protein. Cells were lysed in lysis buffer (20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 1% Nonidet P-40 and 10% glycerol) supplemented with a protease inhibitor cocktail (Roche Diagnostics). The lysate was centrifuged at 4 °C for 10 min at 10 000 g and the pellet was discarded. Protein concentrations in the supernatant were measured by the Bradford assay (Bio-Rad). After boiling for 5 min, proteins (20 μg) were separated on 10% SDS–PAGE slab gels in a Mini PROTEAN electrophoretic chamber (Bio-Rad). Proteins resolved in the gels were electroblotted onto nitrocellulose membranes (Bio-Rad). The membranes were blocked 3 h in TTBS (100 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.05% Tween-20) that contained 5% nonfat dry milk and were incubated overnight with 0.4 μg/ml rabbit anti-phospho-CaMKII (Cell Signaling Technology). The immunoreactive band was visualized by incubation for 1 h with 0.04 μg/ml goat anti-rabbit IgG antibody (Chemicon International, Temecula, CA, USA) conjugated to HRP, followed by the Enhanced Western Lighting Chemiluminescence reaction (PerkinElmer Life Sciences, Boston, MA, USA). Blots were stripped in 100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris–HCl, pH 6.7 at 60 °C for 30 min, and reprobed with 0.2 μg/ml rabbit anti-CaMKII antibody and developed in a similar manner to ensure even loading. All blots were then digitalized and the relative level of phospho-CaMKII was normalized against total CaMKII. Oviductal samples without anti-phospho-CaMKII or anti-CaMKII antibody were included as negative controls.

**Immunoelectron microscopy**

A post-embedding immunogold-labelling method that preserves cellular integrity and maintains ER immunogenicity was used (Kessels et al. 1998, Qualmann et al. 2000, Orihuela et al. 2009). Primary smooth muscle cell cultures were fixed in 4% freshly depolymerized paraformaldehyde, 0.5% glutaraldehyde in 1 M phosphate buffer pH 7.4 containing 0.1 M sucrose, 1% DMSO and 1% CaCl₂ for 2–4 h at room temperature. The fixed samples were washed three times with phosphate buffer, dehydrated in a graded ethanol series and infiltrated with LR Gold (Plano, München, Germany). Subsequently, the samples were transferred to gelatin capsules filled with 0.8% (w/v) benzoyl peroxide in LR Gold and kept for polymerization at a pressure of 500 mmHg. The blocks were cured for 1–2 days at room temperature before sectioning with a Sorvall-2000 ultramicrotome using a diamond knife. The sections (50–80 nm) were mounted on formvar-coated nickel grids and incubated on droplets of 0.1 M glycine in PBS pH 7.6, and subsequently blocked with 1% bovine fetal serum for 2 h at room temperature. The grids were then incubated for 2 h with 4.0 μg/ml rabbit anti-ESR1 (MC-20, Santa Cruz Biotechnology) or 4.0 μg/ml anti-ESR2 (clone 68-4, Chemicon International). After washing with PBS, the preparations were incubated for 1 h with 0.3 μg/ml goat anti-rabbit immunoglobulin conjugated to 10 nm gold particles (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Sections were washed and contrasted with Reynolds stain (Reynolds 1963). All sections were examined using a Phillips-TECNAI 12 BIOTWIN EM Microscope (FEI Company, Hillsboro, OR, USA) at 80 kV. As negative control for ESR1 and ESR2, the primary antibody was replaced by preimmune serum. At least ten areas of 63 μm² from different smooth muscle cells from the primary cultures were photographed and the photomicrographs were digitalized in an iBook computer (Apple Computer, Cupertino, CA, USA), and gold particles present only in the cells were counted using the image analysis Software Adobe Photoshop 7.0 (Adobe Systems) by an observer blinded to the different groups. The results of the immunolabelling are presented as the quotient of the number of gold particles present divided by the area and cell number analysed in three different cultures (Orihuela et al. 2009).

**Immunofluorescence**

Smooth muscle cells were fixed in cold 4% paraformaldehyde in PBS pH 7.4–7.6 for 2 h, transferred to 10% (w/v) saccharose in PBS for 60 min at 4 °C and 30% (w/v) saccharose in PBS at 4 °C overnight. Then, they were blocked with 1% PBS–BSA for 120 min, and incubated with 0.8 μg/ml mouse anti-cytokeratin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 2.5 μg/ml mouse anti-vimentin (Santa Cruz Biotechnology), 0.4 μg/ml mouse anti-α-actin (Santa Cruz Biotechnology) or 5 μg/ml mouse anti-Gαz (Santa Cruz Biotechnology) antibodies. After washing with PBS, the preparations were incubated for 2 h with 0.5 μg/ml Alexa fluor 588-conjugated goat anti-mouse IgG (Invitrogen). Sections were washed and counterstained with 1 μg/ml of Hoechst 33342 (Thermo Scientific, Rockford, IL, USA) washing again and then mounted in Fluoromount G. As negative controls, the primary antibody was replaced by preimmune serum. As positive control for Gαz, we used samples of whole oviducts from rats on day 1 of the estrous cycle (Oróstica et al. 2013, 2014). All sections were visualized with an Optiphot Epifluorescence Microscope (Olympus).

**Real-time PCR**

Total RNA from primary secretory cell cultures was isolated using Trizol Reagent (Invitrogen). One microgram of total RNA of each sample was treated with Dnase 1 Amplification grade (Invitrogen). The single-strand cDNA was synthesized by reverse transcription using the Superscript III Reverse Transcriptase First Strand System for RT-PCR (Invitrogen), according to the manufacturer’s protocol. The Light Cycler instrument (Roche Diagnostics) was used to quantify the relative gene expression of the E₂-target genes c-fos (Nilsson et al. 2001) in the oviductal smooth muscle cells; Gapdh was chosen as the housekeeping gene for load control. The SYBR Green I double-strand DNA binding dye (Roche Diagnostics) was the reagent of choice for these assays. Primers for c-fos were 5'-CCG AGA TTG CCA ATC TAC TG 3' (sense) and 5'-AGA AGG ACG ACA GAC GTG CC 3' (antisense) and for Gapdh were 5'-ACC AGA GTC CAT GCC ATC AC 3' (sense) and 5'-TCC ACC ACC CTG TTG CTC TA 3' (anti sense). All real-time PCR assays were performed in duplicate. The thermal cycling conditions included an initial activation step at 95 °C for 5 min, followed by 40 cycles of denaturizing and annealing-amplification (95 °C for 15 s, 59 °C for 30 s and 72 °C for 30 s) and finally one cycle of melting (60 °C up to 95 °C). The expression of c-fos was determined using the equation:

\[
\text{Fold change} = 2^{(-\Delta\Delta C_T)}
\]

where \(\Delta C_T\) is the difference in cycle threshold value between the target gene and the reference gene.
$Y = 2^{-\Delta Cp}$ (16) where $Y$ is the relative expression, $Cp$ (crossing point) is the cycle in the amplification reaction in which fluorescence begins to be exponential above the background base line, $-\Delta Cp$ is the result of subtracting $Cp$ value of c-los from $Cp$ value of gapdh for each sample. To simplify the presentation of the data, the relative expression values were multiplied by $10^3$ (Livak and Schmittgen 2001).

### Statistical analysis

Data for IP$_3$ and CaMKII assays from cultured oviductal cells were replicated five times for each treatment (for each culture experiment, oviductal cells were recovered from a pool of six different rats). Statistical analysis was performed using a GraphPad Prism 5.0 Software program. All data are presented as mean ± S.E.M. These data followed a non-normal distribution (Kolmogorov–Smirnov test) and significant differences between groups were determined through the use of variance analysis by Friedman’s test with subsequent post-hoc Wilcoxon signed-rank test. Significance was accepted at $P<0.05$. On the other, the quantitative analysis of the ESR1 or ESR2 distribution was subjected to Kruskal-Wallis test, followed by Mann-Whitney’s $U$ tests for pairwise comparisons when overall significance was detected. Significance was accepted at $P<0.05$.

### Results

**E$_2$ increased IP$_3$ production by a nongenomic action in the smooth muscle cells from the rat oviduct**

Primary cultures of smooth cells from rat oviducts, with a purity of 90–95% (Fig. 1), were divided into the following treatment groups: i) ethanol + DMSO, ii) E$_2$ + DMSO, iii) ethanol + ActD and iv) E$_2$ + ActD. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of IP$_3$ as described in the ‘Materials and methods’ section.

Figure 2 shows that in the vehicle group, IP$_3$ production ranged from 114.1 ± 30.3 to 123.2 ± 26.7 fmol/µg of protein while in the E$_2$-treated group it was increased at 6 h (523.2 ± 66.7 fmol/µg of protein) but not at 1, 3 or 9 h. Administration of ActD alone or concomitant with E$_2$ did not affect the time-course of IP$_3$ production in the control or in the E$_2$-treated group.

**ESR1 and ESR2 are localized in association with the plasma membrane of the oviductal smooth muscle cells**

Primary cultures of smooth muscle cells from rat oviducts with no treatment were processed by immunoelectron microscopy using specific antibodies for ESR1 and ESR2. Figure 3 shows that in the control groups, the IP$_3$ level ranged from 151.6 ± 26.3 to 193.5 ± 37.3 fmol/µg of protein, while in the E$_2$-treated groups it ranged from 488.3 ± 59.7 to 599.4 ± 71.3 fmol/µg of protein. Administration of ICI 182780 or ET-18-OCH$_3$ alone did not affect the basal IP$_3$ production although blocked the E$_2$-simulated IP$_3$ increase at 6 h.

**Activation of ESR1 but not ESR2 mimic the effect of E$_2$ on the IP$_3$ production in the oviductal smooth muscle cells**

Primary cultures of smooth muscle cells from rat oviducts were treated with DMSO, PPT (10$^{-7}$–10$^{-5}$ M) or DPN (10$^{-7}$–10$^{-5}$ M) during 0, 1, 3, 6 or 9 h and processed to measure the concentration of IP$_3$ as described in the ‘Materials and methods’ section. Other smooth muscle cells cultures were also treated with

---

**Figure 1** Expression of α-actin in primary cultures of smooth muscle cells from rat oviducts. Representative photomicrographs of primary cultures of smooth muscle cells from rat oviducts were processed by immunofluorescence microscopy to detect expression of cytokeratin, vimentin or α-actin. Note that and α-actin (red) were only expressed in smooth muscle cells. Nuclei were stained with Hoechst 33342 (blue). Negative controls of the immunoreactivity were incubated with preimmune serum.
DMSO or DPN 10^{-7} M and 6 h later processed by Real-Time PCR to determine the mRNA level of c-fos.

Figure 5 shows that in the control group, the IP_3 production ranged from 120.3 ± 44.1 to 148.5 ± 50.6 fmol/μg of protein while treatment with PPT increased IP_3 level in a dose-dependent manner at 6 h without any effect at 0, 1, 3 or 9 h. On the other hand, administration of DPN had no effect on the IP_3 level at any time or concentration studied (Fig. 5), although it increased the mRNA level of c-fos (control: 51.4 ± 11.3 vs DPN: 210.7 ± 36.2 relative expression, n=5).

**G_{α_1} protein is not required for the IP_3 production increase induced by E_2 in the oviductal smooth muscle cells**

Primary cultures of smooth cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) E_2 + DMSO, iii) ethanol + PTX and iv) E_2 + PTX. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of IP_3 as described in the ‘Materials and methods’ section. Note that ActD did not block the effect of E_2 on the IP_3 level. This experiment consisted of five replicates.

**E_2 induced activation of CaMKII in the oviductal smooth muscle cells**

Primary cultures from rat oviductal secretory cells were treated with ethanol or E_2 10^{-9} M and 6.5 h later the level of phosphorylated CaMKII (p-CaMKII) was assessed by immunoblot. As E_2 increases the IP_3 level at 6 h after order to assess the immunoreactivity of G_{α_1} in these cells. In addition, we used sections of whole oviducts from rats on day 1 of the oestrous cycle to corroborate presence of G_{α_1} in the oviductal tissues. This experiment was replicated five times.

Figure 6B shows that immunoreactivity of G_{α_1} was not found either in the primary cultures of the oviductal smooth muscle cells or in the myosalpinx layer of the whole oviduct. However, G_{α_1} was expressed in the endosalpinx of the rat oviduct as previously reported by Oróstica et al. (2014).

**G_{α_1} protein is not expressed in the smooth muscle cells from the rat oviduct**

Primary cultures from smooth muscle cells from rat oviducts were processed by immunofluorescence in
treatment, we consider that 6.5 h is a reasonable time to evaluate activation of CaMKII downstream of the IP₃ increase.

Figure 7 shows that E₂ increased the level of p-CaMKII in comparison with the vehicle group.

Discussion
The contribution of the different cell phenotypes of the rat oviduct on the E₂ nongenomic pathway associated with the cAMP-IP₃ signalling and involved in the accelerated egg transport is recently being disclosed (reviewed in Orihuela et al. (2013)). Here we show that E₂ increased IP₃ levels in the oviductal smooth muscle cells by a nongenomic mechanism because suppression of mRNA and protein synthesis by ActD did not prevent the effect of E₂ on the IP₃ level. Moreover, the E₂ nongenomic pathway that increases IP₃ requires activation of ER and PLC since blockade of ER by ICI 182780 and PLC by ET-18-OCH₃ reverted the E₂-induced IP₃ increase in the oviductal smooth muscle cells. Previous works have shown that some E₂ nongenomic pathways are associated with changes in the turnover of inositol lipids that generates IP₃ from the hydrolysis of phosphatidylinositol 4,5-biphosphate in several cell systems (Kisielewska et al. 1996, 1997, Razandi et al. 1999, Ariazi et al. 2010). Our findings show for the first time that a nongenomic action of E₂ associated with PLC-IP₃ signalling is also present in the smooth muscle cells of the mammalian oviduct. The effect of E₂ on the IP₃ level occurred from 6 h and declined at 9 h, indicating a transient action on the PLC-IP₃ signalling in the smooth muscle cells. A rapid turnover of IP₃, inactivation of PLC or down-regulation of ER could explain the lack

Figure 4 Subcellular localization of ESR1 and ESR2 in the primary cultures of smooth muscle cells from rat oviducts. (A) Representative photomicrographs of primary cultures of smooth muscle cells from rat oviducts processed by immunoelectron microscopy with gold labeled-antibodies for ESR1 or ESR2. Arrows show gold particle labeling localized to the plasma membrane (PM), cytoplasm (C) and nucleus (N). Negative controls of the immunoreactivity were incubated with preimmune serum. ICS = intercellular space. (B) Density of gold particles (X±SE) observed for ESR1 and ESR2 in the plasma membrane (PM), cytoplasm (C) and nucleus (N). Note that density of ESR1 and ESR2 in the PM were lower than in the C and N. a≠b≠c, P<0.05. Replicates of this experiment consisted of 12 cells from three different cell cultures (four individuals cells/well plate).

Figure 5 Effect of selective agonists for ESR1 (PPT) or ESR2 (DPN) on the IP₃ level in primary cultures of smooth muscle cells from rat oviducts. Primary cultures of smooth muscle cells from rat oviducts were treated with DMSO, 10⁻⁷ M PPT or DPN during 0, 1, 3, 6 or 9 h and processed to measure the concentration of IP₃ as described in the ‘Materials and methods’ section. Note that PPT increased the IP₃ level in a concentration-dependent manner at 6 h after treatment while DPN had no any effect. This experiment consisted of five replicates. *P<0.05, **P<0.02 and ***P<0.01 with respect to each treatment group.
1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of IP3 as described in the ‘Materials and methods’ section. Note that PTX did not block the effect of E2 on the IP3 level. This experiment consisted of five replicates. *Note that Gα (red) is only expressed in the endosalpinx (mainly composed of epithelium) layer of the whole oviduct. Nuclei were stained with Hoechst 33342 (blue). Negative controls of the immunoreactivity were incubated with preimmune serum. L, lumen; E, endosalpinx; M, myosalpinx.

Figure 6 Effect of PTX on E2-induced IP3 increase and expression of Gα in the oviductal smooth muscle cells. (A) Primary cultures of smooth muscle cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) E2 + DMSO, iii) ethanol + PTX and iv) E2 + PTX. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of IP3 as described in the ‘Materials and methods’ section. Note that PTX did not block the effect of E2 on the IP3 level. This experiment consisted of five replicates. *P<0.05 with respect to each treatment group. (B) Representative photomicrographs obtained from primary cultures of oviductal smooth muscle cells or whole oviduct processed by immunofluorescence to detect expression of Gα protein. Note that Gai (red) is only expressed in the endosalpinx (mainly composed of epithelium) layer of the whole oviduct. Nuclei were stained with Hoechst 33342 (blue). Negative controls of the immunoreactivity were incubated with preimmune serum. L, lumen; E, endosalpinx; M, myosalpinx.

of effect E2 on the IP3 level at 9 h; however, this remains to be determined.

In contrast to other reports that showed a rapid increase of IP3 by E2 in rat vaginal epithelial cells and HEPG2 cells (Singh & Gupta 1997, Marino et al. 1998), we found that E2 has a time of latency of 6 h to exert its effects on the IP3 production in the smooth muscle cells from the rat oviduct. Differences in the expression of the ER isoforms or in the signalling pathways between the different cell phenotypes may explain the delayed response to E2 in the smooth muscle cells. We postulate that the E2 nongenomic action that increases IP3 appears as a secondary response to intracellular changes localized upstream of PLC activation in the oviductal smooth muscle cells. According to this assumption, we have recently shown that E2-induced IP3 increase is preceded by a cAMP decrease in smooth muscle cells from the rat oviduct (Oróstica et al. 2014). Alternatively, E2 may be first metabolized into 2-methoxyestradiol to increase IP3 production in the oviductal smooth muscle cells. In this context, various biological effects of E2 including regulation of egg transport in the rat oviduct or modulation of the antihypertensive and neuroprotective effects of E2, requires previous conversion from E2 to 2ME in its target organs (reviewed in Dubey & Jackson (2001) and Parada-Bustamante et al. (2015)).

Inhibition of the ER activity did not affect basal IP3 production in the oviductal smooth muscle cells, indicating that other ER-independent signalling pathways are acting to state basal IP3 level. In accordance with this idea, various signalling pathways such as Angiotensin-II, arachidonic acid, endothelin-1 and norepinephrine regulate production of IP3 (reviewed in Bolton (2006)). On the other hand, ET-18-OCH3 alone had no effect on the IP3 production in the oviductal smooth muscle cells. Since 13 mammal PLC subtypes have actually been reported (Rhee 2001), it is probable that basal IP3 production depends on an ET-18-OCH3-insensitive PLC. In this context, it has been found that ET-18-OCH3 is more effective at inhibiting membrane-associated PLC-β1 than PLC-γ1 localized in the cytosol of human fibroblasts (Powis et al. 1992).

Physiological effects of E2 are mainly influenced by the differential distribution of ESR1 and ESR2 in its target organs. Our results showing localization of ESR1 and ESR2 in the cell membrane, cytoplasm and nucleus

Figure 7 Effect of E2 on the CaMKII activation in primary cultures of smooth muscle cells from rat oviducts. Primary cultures of smooth muscle cells from rat oviducts were treated with ethanol or E2 and 6.5 h later CaMKII activation was determined by western blot and densitometry using anti-phospho-CaMKII or total CaMKII antibodies. Note that E2 treatment increased the relative phosphorylation of CaMKII in the smooth muscle cells. This experiment consisted of five replicates. *P<0.05 with respect to the control group.
GTPases participate in the activation of the PLC-α and establish the mechanism by which the E₂-ER complex from the rat oviduct, further studies are necessary to indicate compatible localization of both ER subtypes with genomic and nongenomic actions of E₂ in the smooth muscle cells of the rat oviduct. These findings corroborate previous studies on the presence of ER in nuclear and extranuclear regions in the rat oviduct epithelium cells (Orihuela et al. 2009) and reinforce the concept that signalling pathways associated with E₂ nongenomic and genomic actions operate in the mammalian oviduct (Parada-Bustamante et al. 2010, 2012, Orihuela et al. 2013). The fact that a lower proportion of ESR1 and ESR2 was found in the plasma membrane compared with those present in the cytoplasm and nucleus of the oviductal smooth muscle cells is consistent with the notion that only a small pool of ER is responsible for the E₂ nongenomic actions (reviewed in Watson & Gametchu 2003 and Wehling et al. 2006).

The functional relevance of ER subtypes on the E₂-induced IP₃ increase in the oviductal smooth muscle cells was investigated utilizing selective agonists for ESR1 (Harris et al. 2002) or ESR2 (Frasor et al. 2003). We found that activation of ESR1 by PPT mimicked the effect of E₂ on the IP₃ levels while activation of ESR2 by DPN had no effect. This suggests that the nongenomic pathway by which E₂ increases IP₃ in the oviductal smooth muscle cells operates through ESR1 activation. This is in keeping with previous works showing that the nongenomic actions of estrogenic compounds require activation of ESR1 (reviewed in Marino et al. 2006), Moenter & Chu (2012) and Watson et al. (2014)). On the other hand, the E₂ nongenomic pathways associated with activation of ESR2 in the smooth muscle cells of the rat oviduct needs to be disclosed.

It has been documented that the Gα₁ subclass is the G protein most often linked with the E₂ nongenomic actions coupled with a presumptive ESR1 localized in extranuclear sites (Wyckoff et al. 2001, Kumar et al. 2007, Lin et al. 2011, Watson et al. 2012). However, the ADP-ribosylating agent pertussis toxin did not block the effect of E₂ on the IP₃ production in the smooth muscle cells, suggesting that heterotrimeric Gα₁-type proteins are not required for this E₂ nongenomic action. This is corroborated by the fact that the Gα₁ protein was not expressed either in the primary cultures of smooth muscle cells or in the myosalpinx layer (mainly composed of smooth muscle fibers) of whole oviducts from estrous rats. Gαᵢ₁, Gα₁₆ and Gα₁₇-coupled receptors and tyrosine kinase receptors also activate PLC subtypes in a variety of cell systems (Zhu & Birnbaumer 1996, Yang et al. 2013). Furthermore, small Rho and Ras GTases participate in the activation of the PLC-α (Yang et al. 2013). Since differential expression of PLC subtypes and G proteins is unknown in the smooth muscle cells from the rat oviduct, further studies are necessary to establish the mechanism by which the E₂-ER complex activate PLC in these cells.

Previous reports have shown that the cytokine Tumour Necrosis Factor-α (TNF-α) stimulated production and release of contractile mediators as prostaglandins, endothelins and nitric oxide in the bovine oviduct (Wijayagunawardane & Miyamoto 2004, Szőtyek et al. 2011). Moreover, TNF-α modulates the effect of E₂ on the oviductal egg transport and TNF-α receptors are present in the rat oviduct (Oróstica et al. 2013). It is probable that the E₂ nongenomic pathway that increases IP₃ could be associated with TNF-α signalling in the oviductual smooth muscle cells, but this needs to be further explored.

It is well known that binding of IP₃ to its specific receptors (types 1, 2 and 3) localized on the intracellular compartments (nucleus, plasma membrane or endoplasmic reticulum) participates in Ca²⁺ signalling by mediating intracellular Ca²⁺ release, which results in activation of proteins associated with smooth muscle contraction (Kamm & Stull 1985, Kim et al. 2000). In this context, CaMKII is a serine/threonine kinase that is activated by Ca²⁺ and Calmodulin and it is implicated in regulation of vascular tone through phosphorylation of contractile proteins, including myosin light chain kinase (MLCK), the 20-kd myosin light chain (LC20), phospholipase-α2 and the α-subunit of Ca²⁺ channel (reviewed in Kim et al. 2008)). Furthermore, the synthetic estrogen, estradiol benzoate, is able to stimulate CaMKII activity in mouse hippocampus by a nongenomic mechanism (Sawai et al. 2002). Similarly, we found that E₂ increased CaMKII phosphorylation in the smooth muscle cells of the rat oviduct. To our knowledge, this is the first time that an effect of E₂ on the activity of CaMKII in the mammalian oviduct is reported. As E₂ activated CaMKII presumably downstream of the IP₃ increase, it is probable that this enzyme could be part of the PLC-IP₃ signalling cascades induced by estrogens to activate nongenomic actions in smooth muscle cells.

Several works have reported that E₂ exerts its vasoprotective actions regulating the contractile tone of the vascular, airway and myometrium smooth muscle cells (Kisielewska et al. 1996, Townsend et al. 2010, 2012, Cairrão et al. 2012, Holm et al. 2013). These effects are mainly associated with changes in the intracellular Ca²⁺ mobilization associated with cAMP and IP₃ signalling pathways (Kisielewska et al. 1996, 1997, Townsend et al. 2010, 2012). Since E₂ increases the frequency of myosalpinx contractions that accelerate oviductal egg transport in the rat (Moore & Croxatto 1988a,b), we can speculate that this E₂ effect involves IP₃ production and activation of CaMKII in the smooth muscle cells. This E₂ effect on the oviductal egg movement could be involved in the very early maternal–embryo interactions that occur in the uterine tissues necessary to the embryo implantation (Gomez & Muñoz 2015).

In summary, we have found that E₂ increases IP₃ by a nongenomic action operated by ESR1, and that involves activation of PLC in the smooth muscle cells of the rat oviduct. Furthermore, E₂ activates CaMKII presumably downstream of the IP₃ increase in the oviductual smooth muscle cells, suggesting that IP₃ and CaMKII are
involved in the contractile activity of the oviduct. These findings provide new evidence to understand the molecular mechanisms underlying the role of smooth muscle cells on the E2 nongenomic action that accelerates egg transport in the rat oviduct.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work received financial support from grants FONDECYT # 1110662, Proyecto BASAL FBO-07 and Proyectos Basales y Vicerrectoría de Investigación, Desarrollo e Innovación.

References


Kessels MM, Qualmann B, Thole HH & Sierra WD 1998 Subcellular localization of estradiol receptor in MCF7 cells studied with nanogold-labelled antibody fragments. European Journal of Histochemistry 42 259–270.


www.reproduction-online.org


339 Estradiol nongenomic action on the IP3 level


Watson CS & Gametchu B 2003 Proteins of multiple classes may participate in nongenomic steroid actions. Experimental Biology and Medicine 228 1272–1281.


Received 25 March 2015
First decision 24 April 2015
Revised manuscript received 6 July 2015
Accepted 9 July 2015